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13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to develop a diagnostic blood test for breast cancer. The concept is based on the premise that breast cancers shed sufficient quantities of DNA into plasma to be detected using methylation-specific PCR. Initial testing of the method was disappointing because relatively few cancers were detected in serum or plasma from documented breast cancer patients. We modified our methodology to greatly increase the sensitivity of the assay. Concurrently, we tested a larger panel of genes for methylation in breast cancers of various histologic types. The improvement of sensitivity resulted in some loss of specificity, with positive methylation signals detected in white blood cells and normal breast from some individuals. This problem appeared to be somewhat biased toward particular genes, such as BrCA1. Using the modified MSP detection method and a panel of 4 sensitive and relatively specific gene markers, we were then able to detect positive signals in plasma for 21 of 31 cases (62%) with methylation of at least one of the genes in the tumor tissue. Overall, this project has demonstrated potential for using methylation specific PCR to detect breast cancer derived DNA in patients' blood. However, the project has also identified potential issues with regard to sensitivity and specificity that would need to be addressed before application as a clinical assay.

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INTRODUCTION

The purpose of this study is to develop a diagnostic blood test for breast cancer. The concept is based on the premise that breast cancers shed sufficient quantities of DNA into plasma to be detected using methylation-specific PCR.

During the early phases of this study, a small number of serum samples from breast cancer patients were tested for methylation of genes previously determined to be methylated in the cancer tissue. Disappointingly, methylation was detected in only two of these serum samples. Therefore, in the third year of the project, we revised our methodology to increase our sensitivity for detection of MSP products. This modified protocol did increase sensitivity, but also resulted in some loss of specificity, with positive signals seen occasionally in DNA from normal white blood cells and normal breast epithelium. However, using a restricted set of relatively specific (for cancer detection) genes, we conducted a second analysis on plasma samples from 34 breast cancer patients. Overall, 33 of 34 (97%) tumors had methylation of at least one of these genes, and 21 of these 31 cases (62%) of the corresponding plasma DNA specimens had methylation of at least one of the genes. Although this method has some limitations of specificity and sensitivity, it does have potential for further consideration as an approach for breast cancer diagnosis.

BODY

The overall progress of this project is summarized below:

1. Increasing sensitivity for detection of hypermethylation by fluorescent labeling.
We tested various approaches to increasing the sensitivity of the methylation specific PCR (MSP) method (1), including increased numbers of PCR cycle, increased amounts of template DNA, and increased levels of primers. None of these approaches increased sensitivity without producing non-specific PCR products.

We also investigated the use of a fluorescent detection system (Gel-Star), which does help in the recognition of relatively weak PCR products. Overall, the use of this fluorescent detection system increases the number of cases scored as methylated (for any particular gene) by an average of about 25%.

2. Profiling the spectrum of methylation in major classes of breast cancer
Previous studies have generally considered methylation of individual genes in breast cancer. We have now evaluated methylation of 12 different genes in 109 cases of breast cancer, including tumors representing ductal, mucinous, and lobular morphological types. Representative MSP results are shown in the figure below for 3 genes: RARβ, cyclin D2 (CD2), and HIN-1 (2-4). A summary of results for all 12 genes is shown in figure 2.

Figure 1: Analysis of methylation in breast cancer samples using MSP and fluorescent detection. A set of breast cancer samples was analyzed for the RARβ, CD2, and HIN-1 genes. These results are included in the summary shown in figure 2.
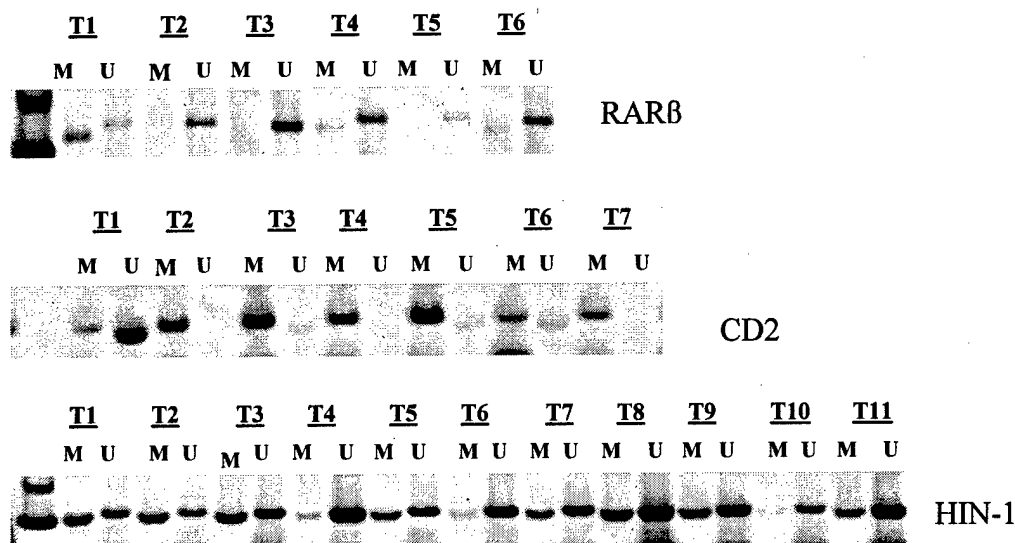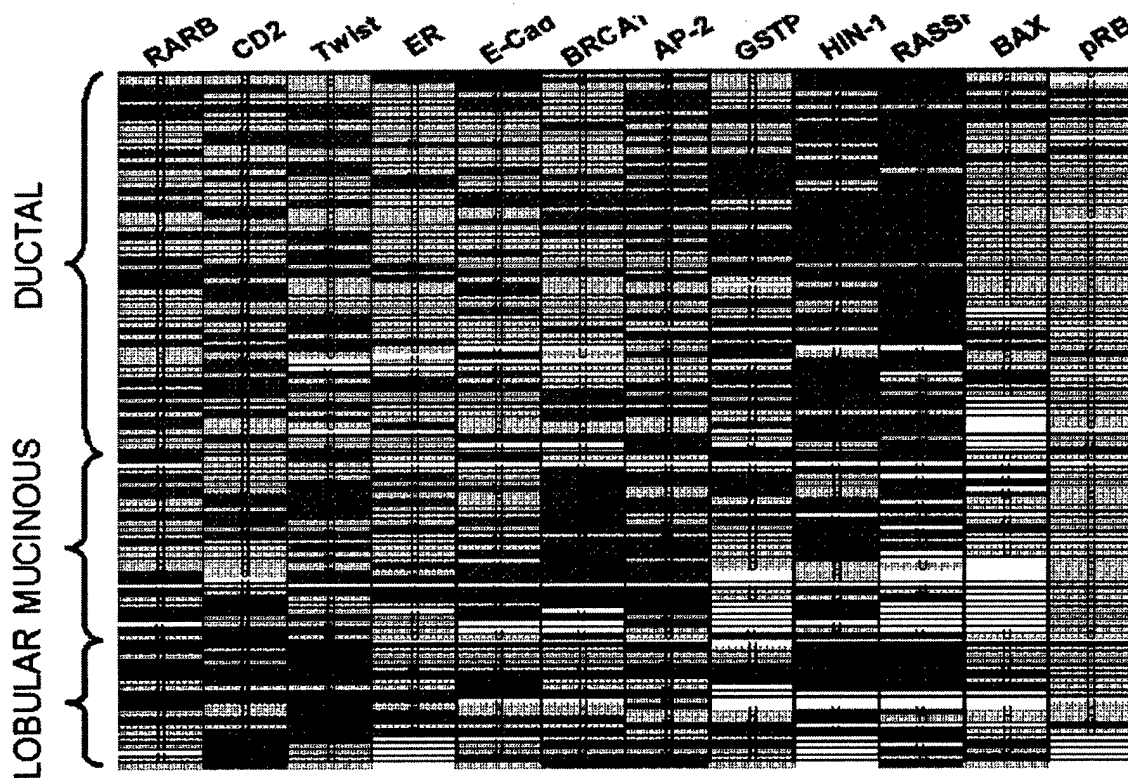

Figure 2: Summary of methylation changes measured in breast cancer samples using MSP and fluorescent detection. A set of breast cancer samples was analyzed for the 12 different genes. Methylation is symbolized by a dark box and absence of methylation (unmethylated) is symbolized by a light tan bar..

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It is important to note that >95% of breast cancers have methylation of HIN-1, RASSF1A, or both. The e-cadherin gene, proposed to be a candidate marker in our original application, is much less frequently methylated that these other genes.

We have analyzed this data to determine whether any patterns of methylation correlate with histologic classification. One gene, BrCa1, is methylated at a significantly higher frequency in mucinous cancers than other types of breast cancer, consistent with previous findings (5). Overall, no histologic type of breast cancer has distinctly higher or lower overall levels of methylation, and we find no evidence for a CpG island methylator phenotype of the type reported for colorectal cancer (6).

3. Detection of methylation of marker genes in normal breast and peripheral blood cells.

The utility of measuring methylation for detection of breast cancer is dependant upon the specificity of a positive reaction for the diagnosis of cancer. Therefore, we analyzed normal blood mononuclear cells and normal mammary epithelial cells for methylation using the same high-sensitivity fluorescent detection system used to analyze breast cancer samples. Representative analyses are shown in figure 3 below and the data is summarized in figure 4.
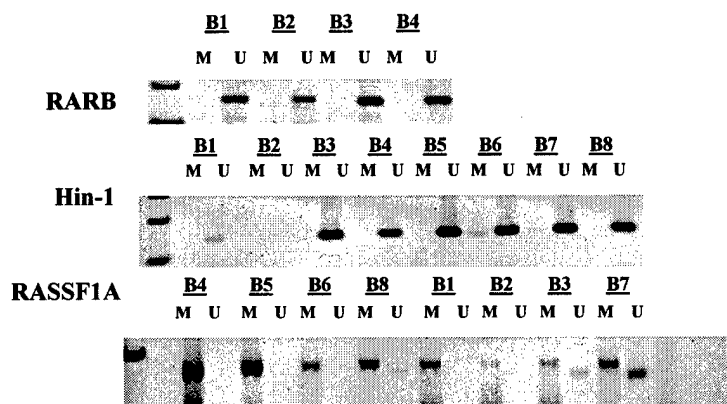

Figure 3: Detection of methylation in normal peripheral blood (B) mononuclear cells by MSP, using fluorescent detection methods. Results are shown for RARβ, HIN-1, and RASSF1A genes.

	Cyclin D2	RARB	E-cadherin	BRCA1	Hin-1	RASSF1A
B1	U	U	U	U	U	■
B2	■	■	■	U		U
B3	U	U	U	U	■	U
B4				■	U	U
B5				■	U	U
B6				U	■	U
B7	U	U	U	U	U	■
B8				U	U	U
Methylation rate (%	25	25	25	25	28.6	25

	Cyclin D2	RARB	E-cadherin	BRCA1	ER	RASSF1A
N1	U		U	■	U	
N2	U		U	■		
N3	U	U		■		U
N4	U	U	U	■	U	U
N5	U	U	U	■	U	U
ethylation rate (	0	0	0	100	0	0

Figure 4: Methylation of genes in normal blood (B) mononuclear cells and normal mammary epithelial cells (N).

These data suggest that the high-sensitivity detection system does have a draw-back of decreased specificity for detection of cancer, compared to conventional methods.

4. Detection of cancer-derived methylated DNA in plasma of breast cancer patients

Work on this project has continued beyond the funding period, and our most recent work does show some promise for detecting breast cancer-derived DNA in plasma. Thirty-four invasive breast cancer patients were examined for aberrant methylation in tumor and plasma DNA using MSP for RARβ, cyclin D2, twist, and HIN1 genes. Overall, 33 of 34 (97%) tumors had methylation of at least one of these genes, and 21 of these 31 cases (62%) of the corresponding plasma DNA specimens had methylation of at least one of the genes. These data are summarized in figure 5 below.

This work remains ongoing, although it is clear that the assay does have the ability to detect cancer-derived DNA in plasma of breast cancer patients. Additional work is being conducted to determine the relationship of tumor size and stage to detection sensitivity, and the overall specificity of a finding of methylated DNA to the diagnosis of cancer.

Genes	Hin-1		RARβ		Cyclin D2		Twist	
Case	Tumor	Plasma	Tumor	Plasma	Tumor	Plasma	Tumor	Plasma
1	U	U	U	U	■	U	U	U
4	■	■	U	U	■	U	U	U
5	■	■	■	U	U	U	U	U
6	■	U	■	U	■	U	■	U
7	U	U	■	U	■	U	■	■
8	■	U	U	U	U	U	U	U
10	■	■	U	U	U	U	■	U
11	■	■	■	U	■	U	■	U
12	■	U	■	U	U	U	■	U
13	■	U	■	U	U	U	U	U
15	U	U	U	U	U	U	U	U
16	■	■	■	U	U	U	■	U
17	■	U	U	U	U	U	■	U
18	■	U	■	U	■	U	■	U
19	■	U	■	■	■	U	U	U
20	■	■	■	U	■	U	U	U
21	■	■	■	U	U	U	U	U
22	■	■	■	U	U	U	■	U
23	■	■	■	U	U	U	■	U
24	U	U	■	U	U	U	U	U
25	■	■	U	U	■	U	U	U
26	■	■	U	U	■	U	U	U
27	■	■	U	U	■	U	■	U
28	U	U	■	U	U	U	U	U
29	■	U	■	U	■	U	■	U
30	■	U	■	U	■	U	■	U
31	■	U	U	U	U	U	U	U
32	■	U	U	U	U	U	U	U
33	U	U	U	U	■	U	■	U
34	■	U	U	U	U	U	U	U
35	■	■	U	U	U	U	■	U
36	U	■	■	U	U	U	■	U
37	■	U	■	U	■	■	U	U
38	U	■	U	U	■	■	U	U

Fig. 5 Gene methylation profiles of primary tumor tissues and paired plasma samples in patients with invasive breast carcinomas. Each row represents MSP results from an individual case. Results were scored as methylated (dark box) or unmethylted (light box).

KEY RESEARCH ACCOMPLISHMENTS

- Methods enhanced for increased detection of methylation.
- Methylation patterns of breast cancer profiled for 12 genes.
- Genes with most frequent methylation identified.
- Non-specific methylation of normal blood cells and normal mammary cells found.
- Methylation of DNA in plasma from breast cancer patients found

REPORTABLE OUTCOMES

The work on methylation profiles of breast cancer has been presented as a poster and platform presentation at the DOD Era of Hope Meeting in Orlando, Florida, September 2002. A manuscript describing the profile of methylation in breast cancer has been submitted (in revised form). Other manuscripts are expected to be prepared and submitted within the year, including one describing methylation of genes in normal mammary cells, and another on detection of methylated DNA in plasma of breast cancer patients.

CONCLUSIONS

Our findings support the proposed approach to develop a blood test to detect breast cancer. However, the finding of some methylation in normal tissues suggests that there will be limits on the sensitivity of this approach.

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